

Mechanisms of Action of Okadaic Acid Class Tumor Promoters on Mouse Skin

by Hirota Fujiki,* Masami Suganuma,* Seiji Yoshizawa,*
Shinji Nishiwaki,* Boonsong Winyar,* and Takashi
Sugimura†

Okadaic acid, dinophysistoxin-1 (35-methylokadaic acid), and calyculin A are the okadaic acid class of non-12-*O*-tetradecanoylphorbol-13-acetate (TPA)-type tumor promoters, which do not bind to the phorbol ester receptors in cell membranes or activate protein kinase C *in vitro*. They have potent tumor-promoting activities on mouse skin, as strong as TPA-type tumor promoters, such as TPA, teleocidin, and aplysiatoxin. DNA samples isolated from tumors induced by dimethylbenz[*a*]anthracene and each of the okadaic acid class tumor promoters had the same mutation at the second nucleotide of codon 61 (CAA to CTA) in the *c-H-ras* gene. Okadaic acid receptors, protein phosphatases 1 and 2A, are present in the particulate as well as cytosolic fractions of various mouse tissues. The apparent "activation" of protein kinases by the okadaic acid class tumor promoters, after their incubation with ³²P-ATP, protein kinases, and protein phosphatases, was observed. This activation was caused by inhibition of protein phosphatases 1 and 2A by the okadaic acid class tumor promoters. Treatment of primary human fibroblasts and human keratinocytes with the okadaic acid class tumor promoters induced the hyperphosphorylation of a 60-kDa protein in nuclear and cytosolic fractions, due to the inhibition of protein phosphatases. The 60-kDa protein is a proteolytic fragment of nucleolin, a major nonhistone protein and is designated as "N-60." The mechanisms of action of the okadaic acid class tumor promoters are discussed with emphasis on the inhibition of protein phosphatase activity.

Introduction

The aim of our research on tumor promotion is to find new tumor promoters on mouse skin and to understand the mechanisms of action of tumor promoters, the structures of which are varied. Based on these studies, we would like to clarify the process of tumor promotion that might take place in human cancer cells (1).

So far we have found 25 new tumor promoters that are structurally different from 12-*O*-tetradecanoylphorbol-13-acetate (TPA). These new tumor promoters were classified into TPA-type tumor promoters and non-TPA type tumor promoters, depending on their ability to bind to the phorbol ester receptors. TPA-type tumor promoters act through the activation of protein kinase C, whereas non-TPA type tumor promoters do not mediate through protein kinase C (2).

Since the tumor-promoting activity of the okadaic acid class compounds is as strong as TPA, teleocidin, and aplysiatoxin, and the strongest of the other non-TPA

type tumor promoters, such as palytoxin, thapsigargin, and staurosporine (3,4), we started to study how the okadaic acid class tumor promoters act on cells (5-7). We called the okadaic acid class tumor promoters those which bound to the okadaic acid receptors, protein phosphatases 1 and 2A (1).

Okadaic Acid Class Tumor Promoters

Okadaic acid, dinophysistoxin-1 (35-methylokadaic acid), and calyculin A are the new okadaic acid class tumor promoters (Fig. 1). Okadaic acid and dinophys-

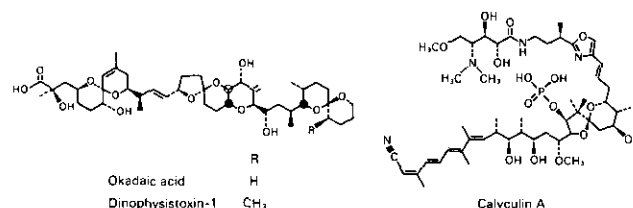


FIGURE 1. Structures of the okadaic acid class tumor promoters, okadaic acid, dinophysistoxin-1, and calyculin A.

*Cancer Prevention Division, National Cancer Center Research Institute, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104, Japan.

†National Cancer Center, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104, Japan.

Address reprint requests to H. Fujiki, Cancer Prevention Division, National Cancer Center Research Institute, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104, Japan.

istoxin-1 were both isolated from a black sponge, *Halichondria okadaei* (8). Dinophysistoxin-1 was first isolated from the hepatopancreas of the mussel, as a causative agent of diarrhetic shellfish poisoning in Japan (9). Calyculin A was isolated from a sponge, *Discodermia calyx* (10). Although calyculin A is structurally unrelated to okadaic acid, calyculin A bound to the okadaic acid receptors.

Table 1 shows tumor-promoting activity of the three okadaic acid class tumor promoters, okadaic acid, dinophysistoxin-1, and calyculin A. From 1 week after initiation with a single application of 100 μg of 7,12-dimethylbenz[*a*]anthracene (DMBA), 1 μg of each compound of the okadaic acid class was applied to the skin of the back of mice, twice a week. Okadaic acid, dinophysistoxin-1, and calyculin A gave similarly high percentages of tumor-bearing mice and also high average numbers of tumors per mouse, which were similar to results with the most potent TPA-type tumor promoters. These data indicated that a potent tumor-promoting activity could be achieved by the okadaic acid receptors as well as by the phorbol ester receptors.

In collaboration with I. B. Weinstein's group at Columbia University and S. Manam at Merck Sharp & Dohme Research Laboratories, we studied the activation of *c-H-ras* gene in tumors. DNA samples isolated from tumors of the three groups treated with DMBA plus each tumor promoter of the okadaic acid class were analyzed by polymerase chain reaction procedure and DNA sequencing. The mutation of CAA to CTA was found in codon 61 of mouse *c-H-ras* gene in the DNA samples (11). The same mutation of *c-H-ras* gene was reported in mouse skin tumors treated with DMBA plus TPA, teleocidin, aplysiatoxin, chrisarobin, mezerein, and benzoylperoxide (11–13). Thus, hypermutability of codon 61 of *c-H-ras* gene is an essential feature in a two-stage carcinogenesis experiment on mouse skin.

Okadaic Acid Receptors

Because the potency of tumor-promoting activity of the okadaic acid class was comparable to that of the TPA-type tumor promoters, we thought that okadaic acid might bind to a specific receptor (5). Figure 2 demonstrates the specific ^3H -okadaic acid binding to the particulate fraction of mouse skin. This specific ^3H -okadaic acid binding was inhibited by okadaic acid, dinophysistoxin-1, and calyculin A, but not by okadaic acid tetramethyl ether, an inactive compound (data not

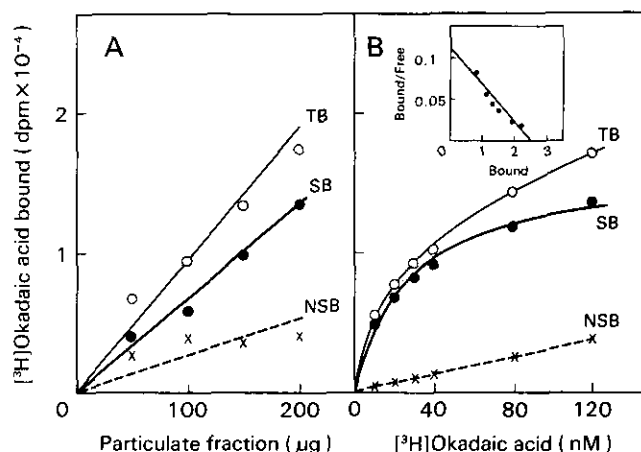


FIGURE 2. Specific binding of ^3H -okadaic acid to the particulate fraction of mouse skin. (A) With 80 nM ^3H -okadaic acid; (B) with 200 nM ^3H -okadaic acid. (TB, \circ) total binding; (SB, \bullet) specific binding; (NSB, \times) nonspecific binding.

shown). Since this specific ^3H -okadaic acid binding was not inhibited by TPA, teleocidin, aplysiatoxin, palytoxin, or thapsigargin, okadaic acid class tumor promoters were thought to bind to their own receptors. The okadaic acid receptors were also found in the cytosolic fraction of mouse skin. Although the conditions of the binding assays of ^3H -okadaic acid to the particulate and cytosolic fractions were not the same, the K_D value of ^3H -okadaic acid of the cytosolic fraction was about 10 times higher than that of the particulate fraction (5).

To better understand the biochemical nature of okadaic acid receptors in the cytosolic fraction, the cytosolic fraction of mouse epidermis and mouse brain were subjected to DEAE-cellulose column chromatography. The specific ^3H -okadaic acid binding fraction from both the cytosolic fractions, which was eluted with 0.2 M NaCl, contained protein phosphatases and protein kinases, the latter of which were distinct from protein kinase C (6). Okadaic acid bound to purified protein phosphatases 1 and 2A (5,6), which catalyze the dephosphorylation of phosphoserine and phosphothreonine of proteins and inhibited protein phosphatase activities (14–16).

Recently, we have demonstrated that a photoaffinity probe of okadaic acid covalently bound to a catalytic

Table 1. Tumor-promoting activities of the okadaic acid class compared with those of TPA-type tumor promoters.

Promoter	Amount of compound/ application, μg (nmole)	Maximal percentage of tumor- bearing mice	Average numbers of tumors/ mouse in week 30
Okadaic acid	1.0 (1.2)	86.7	7.2
Dinophysistoxin-1	1.0 (1.2)	100.0	8.5
Calyculin A	1.0 (1.0)	93.3	4.3
TPA	2.5 (4.1)	100.0	11.0
Teleocidin	2.5 (5.7)	100.0	4.0
Aplysiatoxin	2.5 (4.0)	80.0	3.4

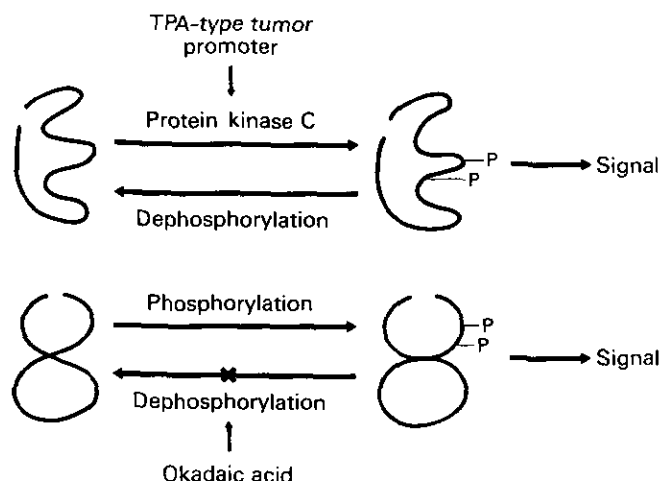


FIGURE 3. Mechanisms of action of the TPA-type tumor promoters and the okadaic acid class tumor promoters.

subunit of protein phosphatase 2A (Fujiki et al., unpublished results). With ^{32}P -ATP, the incubation of protein phosphatases and protein kinases, which are both contained in the binding fraction, achieved a 3-fold activation of protein kinase activity by okadaic acid (6). From these results, we understood that inhibition of protein phosphatases by okadaic acid resulted in the apparent "activation" of protein kinases because okadaic acid itself did not cause any direct effect on the activity of protein kinases (6,7).

As for the target proteins from the apparent "activation" of protein kinases by okadaic acid in the cells, we had previously reported that okadaic acid induced a hyperphosphorylation of a 60-kDa protein in primary human fibroblasts (17). This hyperphosphorylation of a 60-kDa protein (N-60) was also found in human keratinocytes immortalized by human papilloma virus DNA by treatment with the okadaic acid class tumor promoters (data not shown). A hyperphosphorylated N-60, which might be short-lived, becomes a long-lived protein by inhibition of protein phosphatases by okadaic acid. Subsequently, the remaining hyperphosphorylated N-60 is probably involved in the clonal expansion of the initiated cells (1,7,11).

The mechanisms of action of the okadaic acid class tumor promoters are compared to TPA-type tumor promoters (Fig. 3). Okadaic acid inhibits protein phosphatases and accumulates the phosphorylated proteins in larger amounts than in the usual state, which is reflected as the apparent "activation" of protein kinases. The okadaic acid class tumor promoters open up a new understanding of the mechanisms of tumor promotion on mouse skin.

This work was supported in part by Grants-in-Aid for Cancer Research from the Ministry of Education, Science and Culture and the Ministry of Health and Welfare, for a Comprehensive 10-Year Strat-

egy for Cancer Control, Japan, and by grants from the Foundation for Promotion of Cancer Research and the Princess Takamatsu Cancer Research Fund.

REFERENCES

1. Fujiki, H., Suganuma, M., and Sugimura, T. Significance of new environmental tumor promoters. *Environ. Carcinog. Rev.* 7: 1-51 (1989).
2. Fujiki, H., and Sugimura, T. New classes of tumor promoters: teleocidin, aplysiatxin, and palytoxin. *Adv. Cancer Res.* 49: 223-264 (1987).
3. Suganuma, M., Fujiki, H., Suguri, H., Yoshizawa, S., Hirota, M., Nakayasu, M., Ojika, M., Wakamatsu, K., Yamada, K., and Sugimura, T. Okadaic acid: an additional non-phorbol-12-tetradecanoate-13-acetate-type tumor promoter. *Proc. Natl. Acad. Sci. U.S.A.* 85: 1768-1771 (1988).
4. Fujiki, H., Suganuma, M., Suguri, H., Yoshizawa, S., Takagi, K., Uda, N., Wakamatsu, K., Yamada, K., Murata, M., Yasumoto, T., and Sugimura, T. Diarrhetic shellfish toxin, dinophysistoxin-1, is a potent tumor promoter on mouse skin. *Jpn. J. Cancer Res.* 79: 1089-1093 (1988).
5. Suganuma, M., Suttajit, M., Suguri, H., Ojika, M., Yamada, K., and Fujiki, H. Specific binding of okadaic acid, a new tumor promoter in mouse skin. *FEBS Lett.* 250: 615-618 (1989).
6. Sassa, T., Richter, W. W., Uda, N., Suganuma, M., Suguri, H., Yoshizawa, S., Hirota, M., and Fujiki, H. Apparent "activation" of protein kinases by okadaic acid class tumor promoters. *Biochem. Biophys. Res. Commun.* 159: 939-944 (1989).
7. Fujiki, H., Suganuma, M., Sassa, T., Uda, N., Suguri, H., Nishiwaki, S., Yoshizawa, S., Iida, Y., Paik, N. S., and Sugimura, T. Biochemical mechanisms of action of new tumor promoters and anti-tumor promoters. In: *Proceedings of the Federation of Asian and Oceanian Biochemists*, in press.
8. Tachibana, K., Scheuer, P. J., Tsukitani, Y., Kikuchi, H., Van Engen, D., Clardy, J., Gopichand, Y., and Schmitz, F. J. Okadaic acid, a cytotoxic polyether from two marine sponges of the genus *Halichondria*. *J. Am. Chem. Soc.* 103: 2469-2471 (1981).
9. Murata, M., Shimatani, M., Sugitani, H., Oshima, Y., and Yasumoto, T. Isolation and structural elucidation of the causative toxin of the diarrhetic shellfish poisoning. *Bull. Jpn. Soc. Sci. Fish.* 48: 549-552 (1982).
10. Kato, Y., Fusetani, N., Matsunaga, S., Hashimoto, K., Fujita, S., and Furuya, T. Calyculin A, a novel antitumor metabolite from the marine sponge *Discodermia calyx*. *J. Am. Chem. Soc.* 108: 2780-2781 (1986).
11. Fujiki, H., Suganuma, M., Yoshizawa, S., Kanazawa, H., Sugimura, T., Manam, S., Kahn, S. M., Jiang, W., Hoshina, S., and Weinstein, I. B. Codon 61 mutations in the c-Harvey-ras gene in mouse skin tumors induced by 7,12-dimethylbenz(a)anthracene plus okadaic acid class tumor promoters. *Mol. Carcinog.* 2: 184-187 (1989).
12. Quintanilla, M., Brown, K., Ramsden, M., and Balmain, A. Carcinogen-specific mutation and amplification of Ha-ras during mouse skin carcinogenesis. *Nature* 322: 78-80 (1986).
13. Pelling, J. C., Fischer, S. M., Neaders, R., Strawhecker, J., and Schweickert, L. Elevated expression and point mutation of the Ha-ras protooncogene in mouse skin tumors promoted by benzoylperoxide and other promoting agents. *Carcinogenesis* 8: 1481-1484 (1987).
14. Erdödi, F., Rokolya, A., Di Salvo, J., Barany, M., and Barany, K. Effect of okadaic acid on phosphorylation-dephosphorylation of myosin light chain in aortic smooth muscle homogenate. *Biochem. Biophys. Res. Commun.* 153: 156-161 (1988).
15. Hescheler, J., Mieskes, G., Rügge, J. C., Takai, A., and Trautwein, W. Effects of a protein phosphatase inhibitor, okadaic acid, on membrane currents of isolated guinea-pig cardiac myocytes. *Pflügers Arch.* 412: 248-252 (1988).

16. Haystead, T. A. J., Sim, A. T. R., Carling, D., Honnor, R. C., Tsukitani, Y., Cohen, P., and Hardie, D. G. Effects of the tumour promoter okadaic acid on intracellular protein phosphorylation and metabolism. *Nature* 337: 78–81 (1989).
17. Issinger, O. -G., Martin, T., Richter, W. W., Olson, M., and Fujiki, H. Hyperphosphorylation of N-60, a protein structurally and immunologically related to nucleolin after tumour-promoter treatment. *EMBO J.* 7: 1621–1626 (1988).